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(57) Abstract

Improved vaccines which include a nucleotide sequence that encodes immunomodulating protein operably linked to regulatory elements are disclosed. The improved vaccines include DNA vaccines, recombinant vaccines for delivering foreign antigen and live attenuated vaccines. Methods of immunizing individuals are disclosed. Compositions for and methods of treating individuals with autoimmune diseases are disclosed.

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VACCINES, IMMUNOTHERAPEUTICS AND METHODS FOR USING THE SAME

FIELD OF THE INVENTION

The present invention relates to improved vaccines, improved methods for prophylactically and/or therapeutically immunizing individuals against immunogens, and to improved immunotherapeutic compositions and improved immunotherapy methods.

BACKGROUND OF THE INVENTION

This application claims priority to U.S. Provisional Application 60/076,207 filed February 27, 1998 and entitled "Improved Vaccines", which is incorporated herein by reference. Immunotherapy refers to modulating a persons immune responses to impart a desirable therapeutic effect. Immunotherapeutics refer to those compositions which, when administered to an individual, modulate the individual's immune sufficient to decrease symptoms and causes of symptoms brought on by undesirable immune responses or to alleviate symptoms or eliminate/reduce causes of symptoms by increasing desirable immune responses. In some cases, immunotherapy is part of a vaccination protocol in which the individual is administered a vaccine that results in the individual being exposed to an immunogen. In such cases, the immunotherapeutic increases the immune response and/or selectively enhances a portion of the immune response which is desirable to treat or prevent the particular condition, infection or disease. In some cases, immunotherapeutics are delivered free of immunogens. In such cases, the immunotherapeutics are provided to modulate the immune system by either decreasing or suppressing immune responses, enhancing or increasing immune responses, decreasing or suppressing a portion of immune system, enhancing or increasing a portion of the immune system or decreasing or suppressing immune responses, enhancing or increasing immune responses. In some cases, immunotherapeutics include antibodies which when administered *in vivo*, bind to

proteins involved in modulating immune responses. The interaction between antibodies and such proteins results in the alteration of immune responses. If the protein is involved in autoimmune disease, the antibodies can inhibit its activity in that role and reduce or eliminate the symptoms or disease.

Vaccines are useful to immunize individuals against target antigens such as allergens, pathogen antigens or antigens associated with cells involved in human diseases. Antigens associated with cells involved in human diseases include cancer-associated tumor antigens and antigens associated with cells involved in autoimmune diseases.

In designing such vaccines, it has been recognized that vaccines which produce the target antigen in the cell of the vaccinated individual are effective in inducing the cellular arm of the immune system. Specifically, live attenuated vaccines, recombinant vaccines which use avirulent vectors and DNA vaccines all lead to the production of antigens in the cell of the vaccinated individual which results induction of the cellular arm of the immune system. On the other hand, sub-unit vaccines which comprise only proteins and killed or inactivated vaccines, which do induce a humoral response, do not induce good cellular immune responses.

A cellular immune response is often necessary to provide protection against pathogen infection and to provide effective immune-mediated therapy for treatment of pathogen infection, cancer or autoimmune diseases. Accordingly, vaccines which produce the target antigen in the cell of the vaccinated individual such as live attenuated vaccines, recombinant vaccines which use avirulent vectors and DNA vaccines are preferred.

While such vaccines are often effective to immunize individuals prophylactically or therapeutically against pathogen infection or human diseases, there is a need for improved vaccines. There is a need for compositions and methods which produce an enhanced immune response.

SUMMARY OF THE INVENTION

The present invention related compositions which comprise immunomodulating proteins or nucleic acid molecules that encode the same, which

enhance and/or modulate the immune response, as well as methods of using such proteins and nucleic acid molecules. The delivery of immunomodulating proteins is useful for immunotherapy as well as for enhancing or otherwise tailoring immune responses in conjunction with vaccine delivery. An immunomodulating proteins may be: a chemokine including MCP-1, MIP-1α, MIP-1β, IL-8 and RANTES; an adhesion molecule including a selectin such as L-selectin, P-selectin and E-selectin, a mucin-like molecule such as CD34, GlyCAM-1, and MadCAM-1, a member of the integrin family such as LFA-1, VLA-1, Mac-1 and p150.95, a member of the immunoglobulin superfamily such as PECAM, ICAMs e.g. ICAM-1, ICAM-2 and ICAM-3, CD2 and LFA-3; cytokines including M-CSF, G-CSF, GSF, IL-4, mutant forms of IL-18; co-stimulatory molecules such as CD40 and CD40L; growth factors including vascular growth factor, IL-7, nerve growth factor and vascular endothelial growth factor; receptor molecules including Fas, TNF receptor, Flt, Apo-1, p55, WSL-1, DR3, TRAMP, Apo-3, AIR, LARD, NGRF, DR4, DR5, KILLER, TRAIL-R2, TRICK2, DR6;others include Caspase (ICE).

The present invention relates to a plasmid which comprises a nucleotide sequence that encodes an immunomodulating protein operably linked to regulatory elements necessary for expression in eukaryotic cells and a nucleotide sequence that encodes an immunogen operably linked to regulatory elements necessary for expression in eukaryotic cells. The immunogen is preferably a pathogen antigen, a cancer-associated antigen or an antigen linked to cells associated with autoimmune diseases.

The present invention relates to a method of inducing an immune response in an individual against an immunogen comprising the step of administering to an individual, a plasmid which comprises a nucleotide sequence that encodes an immunomodulating protein operably linked to regulatory elements necessary for expression in cells of the individual, and a nucleotide sequence that encodes an immunogen operably linked to regulatory elements necessary for expression in cells of the individual.

The present invention relates to a method of immunizing an individual against a pathogen, cancer or an autoimmune disease comprising the step of administering to an individual, a plasmid which comprises a nucleotide sequence that

encodes an immunomodulating protein operably linked to regulatory elements necessary for expression in cells of the individual, and a nucleotide sequence that encodes an immunogen operably linked to regulatory elements necessary for expression in cells of the individual, wherein the immunogen is a pathogen antigen, a cancer-associated antigen or an antigen linked to cells associated with autoimmune diseases.

The present invention relates to a composition which comprises a first plasmid which comprises a nucleotide sequence that encodes an immunomodulating protein operably linked to regulatory elements necessary for expression in eukaryotic cells and a second plasmid which comprises a nucleotide sequence that encodes an immunogen operably linked to regulatory elements necessary for expression in eukaryotic cells. In some preferred embodiments, the immunogen is a pathogen antigen, a cancer-associated antigen or an antigen linked to cells associated with autoimmune diseases.

The present invention relates to a method of immunizing an individual against a pathogen, cancer or an autoimmune disease comprising the step of administering to an individual, a composition which comprises a first plasmid which comprises a nucleotide sequence that encodes an immunomodulating protein operably linked to regulatory elements necessary for expression in cells of the individual, and a second plasmid which comprises a nucleotide sequence that encodes an immunogen operably linked to regulatory elements necessary for expression in cells of the individual, wherein the immunogen is a pathogen antigen, a cancer-associated antigen or an antigen linked to cells associated with autoimmune diseases.

The present invention relates to a method of inducing an immune response against an immunogen comprising the step of administering to an individual, a composition which comprises a first plasmid which comprises a nucleotide sequence that encodes an immunomodulating protein operably linked to regulatory elements necessary for expression in cells of the individual, and a second plasmid which comprises a nucleotide sequence that encodes an immunogen operably linked to regulatory elements necessary for expression in cells of the individual.

The present invention relates to an improved recombinant vaccine vector which comprises a nucleotide sequence that encodes an immunomodulating protein

operably linked to regulatory elements necessary for expression in eukaryotic cells and a nucleotide sequence that encodes a target antigen operably linked to regulatory elements necessary for expression in eukaryotic cells. In preferred embodiments, the target antigen is a pathogen antigen, a cancer-associated antigen or an antigen linked to cells associated with autoimmune diseases.

The present invention relates to a method of immunizing an individual against a pathogen, cancer or an autoimmune disease comprising the step of administering to an individual, a recombinant vaccine vector which comprises a nucleotide sequence that encodes an immunomodulating protein operably linked to regulatory elements necessary for expression in cells of the individual, and a nucleotide sequence that encodes a target antigen operably linked to regulatory elements necessary for expression in cells of the individual, wherein the target antigen is a pathogen antigen, a cancer-associated antigen or an antigen linked to cells associated with autoimmune diseases.

The present invention relates to a method of inducing an immune response against and target antigen comprising the step of administering to an individual, a recombinant vaccine vector which comprises a nucleotide sequence that encodes an immunomodulating protein operably linked to regulatory elements necessary for expression in cells of the individual, and a nucleotide sequence that encodes a target antigen operably linked to regulatory elements necessary for expression in cells of the individual.

The present invention relates to an improved live, attenuated vaccine which comprises a nucleotide sequence that encodes an immunomodulating protein operably linked to regulatory elements necessary for expression in eukaryotic cells.

The present invention relates to a method of immunizing an individual against a pathogen, cancer or an autoimmune disease comprising the step of administering to an individual, an attenuated vaccine which comprises a nucleotide sequence that encodes an immunomodulating protein operably linked to regulatory elements necessary for expression in cells of the individual.

The present invention relates to a method of inducing an immune response in an individual against an immunogen comprising the step of administering to

an individual, an attenuated vaccine which comprises a nucleotide sequence that encodes an immunomodulating protein operably linked to regulatory elements necessary for expression in cells of the individual.

The present invention relates to compositions and methods for modulating an individual's immune system. The methods of the invention comprise delivering an immunomodulating protein to an individual, either by administration of protein or administration of a nucleotide sequence that encodes an immunomodulating protein as part of an expression vector or other vehicle capable of delivering a nucleotide sequence to an individual in expressible form.

The present invention relates to compositions and methods for treating individuals who have autoimmune diseases. The methods of the invention comprise administering to such individuals, a composition comprising antibodies that specifically bind to chemokines including MCP-1, MIP-1α, MIP-1β, IL-8 and RANTES.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B depict preprocessed and mature IL-18 as discussed in Example 2.

Figure 2 shows the genes for ICAM-1 (pCICAM-1), LFA-3 (pCLFA-3), and VCAM-1 (pCVCAM-1) cloned into the pCDNA3 expression vector.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The invention arises from the discovery that particular proteins enhance and/or modulate the immune response. Accordingly, such proteins may be delivered as immunotherapeutics or as components in a vaccine.

As used herein, the term "immunomodulating proteins" is meant to refer to proteins and nucleic acid molecule expression products according to the present invention which enhance and/or modulate the immune response. Accordingly, immunomodulating proteins may be delivered as immunotherapeutics or as components in a vaccine.

Immunomodulating proteins include chemokines, adhesion molecules, cytokines, co-stimulatory molecules, growth factors, and receptor molecules.

Chemokines that are immunomodulating proteins include MIP-1α, MIP-1β, RANTES, IL-8 and MCP-1.

Adhesion molecules that are immunomodulating proteins include members of the selectin family, mucin-like molecules, members of the integrin family, and members of the immunoglobulin superfamily.

Members of the selectin family that are immunomodulating proteins include L-selectin, P-selectin and E-selectin.

Mucin-like molecules are ligands to members of the selectin family. Mucin-like molecules that are immunomodulating proteins include CD34, GlyCAM-1 and MAdCAM-1.

Members of the integrin family that are immunomodulating proteins include LFA-1, VLA-1 Mac-1 and p150.95.

Members of the immunoglobulin superfamily that are immunomodulating proteins include PECAM, ICAMs, ICAM-1, ICAM-2, ICAM-3, CD2 and LFA-3.

Cytokines that are immunomodulating proteins include M-CSF, GM-CSF, G-CSF, CSF, IL-4, and mutant forms of IL-18 which include a deletion of the first about 35 amino acid residues present on the pro-form of the protein but not the mature form.

Co-stimulatory molecules that are immunomodulating proteins include CD40 and CD40 ligand (CD40L).

Growth factors that are immunomodulating proteins include vascular growth factor, IL-7, nerve growth factor and vascular endothelial growth factor.

Receptor molecules that are immunomodulating proteins include Fas "death gene" expression product, tumor necrosis factor TNF receptor, Flt, Apo-1, p55, WSL-1, DR3, TRAMP, Apo-3, AIR, LARD, NGRF, DR4, DR5, KILLER, TRAIL-R2, TRICK2, DR6;others include Caspase (ICE).

Other molecules include Caspase-1 (ICE).

According to some embodiments of the invention, an immunomodulating protein is delivered by administering a nucleic acid molecule which, when taken up by a cell, is expressed to produce the immunomodulating protein. According to some embodiments of the invention, the immunomodulating protein is delivered by

administering the protein itself. According to some embodiments of the invention, the immunomodulating protein is delivered by administering either nucleic acid molecules or protein. According to some embodiments of the invention, the immunomodulating protein is delivered by administering both nucleic acid molecules and proteins simultaneously.

According to some embodiments of the invention, the immunomodulating protein, either as a protein or a nucleic acid molecule encoding the protein, is administered as a component of or otherwise as a supplement to in conjunction with a vaccine composition. The vaccine may be either a subunit vaccine, a killed vaccine, a live attenuated vaccine, a cell vaccine, a recombinant vaccine or a nucleic acid or DNA vaccine. In the case of a live attenuated vaccine, a cell vaccine, a recombinant vaccine or a nucleic acid or DNA vaccine, the immunomodulating protein may be encoded by the nucleic acid molecules of these vaccines.

Immunomodulating proteins are useful to induce and enhance cytotoxic T cell (CTL) responses, and/or induce and enhance antibody responses, and/or induce and enhance T cell proliferation responses.

Immunomodulating proteins that induce and enhance CTL responses are particularly useful when administered in conjunction or as part of a vaccine against an intracellular pathogens, or against cells associated with autoimmune disease or cancer. Immunomodulating proteins that induce and enhance CTL responses are particularly useful when administered in conjunction with live attenuated vaccines, cell vaccines, recombinant vaccines, and nucleic acid/DNA vaccines. Alternatively, immunomodulating proteins that induce and enhance CTL responses are useful as immunotherapeutics which are administered to patients suffering from cancer or intracellular infection. Immunomodulating proteins that induce and enhance CTL responses are useful when administered to immunocompromised patients.

Immunomodulating proteins that induce and enhance antibody responses are particularly useful when administered in conjunction or as part of a vaccine against bacteria, other extracellular pathogens, or those viruses for which antibody responses are protective such as hepatitis B virus. Immunomodulating proteins that induce and enhance antibody responses are particularly useful when administered in conjunction

with subunit vaccines. Alternatively, immunomodulating proteins that induce and enhance antibody responses are useful as immunotherapeutics which are administered to patients suffering from undesirable CTL immune responses. Such shifting of the patient's immune system reduces the pathology caused by the CTL response. Immunomodulating proteins that induce and enhance antibody responses are useful when administered to immunocompromised patients.

Immunomodulating proteins that induce and enhance T cell proliferation responses are particularly useful when administered in conjunction or as part of vaccines. Alternatively, immunomodulating proteins that induce and enhance T cell proliferation responses are useful as immunotherapeutics. Immunomodulating proteins that induce and enhance T cell proliferation responses are useful when administered to immunocompromised patients.

Chemokines:

The administration of chemokines or nucleic acid molecules that encode chemokines results in an increased expression of chemokines by cells.

MCP-1 is particularly useful in inducing and enhancing CD8+ CTLs.

MIP-1α is particularly useful in the induction of antibodies.

IL-8 is particularly useful in the induction of antibodies, and is a strong inducer of T helper responses.

RANTES induces TH1 as well as CTL responses.

MIP-1β, such as the construct which is been cloned into pCDNA3 to generate pCDNA3- MIP-1β, may also be used.

Adhesion molecules:

Members of the selectin family

L-selectin

P-selectin

E-selectin.

Mucin-like molecules

CD34

GlyCAM-1 such as the construct which has been cloned into pCDNA3 to generate pCDNA3-GlyCAM-1

MadCAM-1.

Members of the integrin family

LFA-1

VLA-1

Mac-1

p150.95

Members of the immunoglobulin superfamily

PECAM

ICAMs

ICAM-1

ICAM-2

ICAM-3

CD2

LFA-3.

Adhesion molecules are most useful when administered as nucleic acid molecules.

Adhesion molecules are most useful when administered as nucleic acid molecules as part of or in conjunction with vaccines, particularly live attenuated vaccines, cell vaccines, recombinant vaccines, and nucleic acid/DNA vaccines.

Adhesion molecules useful when delivered as nucleic acid molecules intratumor or intralesion.

Preferred adhesion molecules include ICAM-1, LFA-3 and E-selectin.

ICAM-1 is best for CTL and proliferation.

Cytokines

M-CSF

G-CSF

CSF

IL-4

mutant forms of IL-18

Co-stimulatory molecules

CD40 such as the construct in which cDNA encoding CD40 is cloned into pCDNA3 to generate pCDNA3-CD40 may be used

CD40L

Growth factors

vascular growth factor such as the construct in which cDNA encoding vascular growth factor is cloned into pCDNA3 to generate pCDNA3-VGF may be used

IL-7

nerve growth factor

vascular endothelial growth factor

Receptor molecules

Fas "death gene" expression product

TNF receptor

Flt

Apo-1

p55

WSL-1

DR3

TRAMP

Apo-3

AIR

LARD

NGRF

DR4

DR5

KILLER

TRAIL-R2

TRICK2

DR6

Other

Caspase (ICE)

Table 1 lists the GENBANK Accession numbers and journal citations for the nucleotide and amino acid sequences for each of the above immunomodulating proteins and for CD86 (B7.2).

DNA vaccines are described in PCT/US90/01515, PCT/US93/02338, PCT/US93/048131, and PCT/US94/00899, and the priority applications cited therein, which are each incorporated herein by reference. In addition to the delivery protocols described in those applications, alternative methods of delivering DNA are described in U.S. Patent Nos. 4,945,050 and 5,036,006, which are both incorporated herein by reference.

Some aspects of the present invention relate to methods of introducing genetic material into the cells of an individual in order to induce immune responses against proteins and peptides which are encoded by the genetic material. The methods comprise the steps of administering to the tissue of said individual, either a single nucleic acid molecule that comprises a nucleotide sequence that encodes a desired peptide or protein and a nucleotide sequence that encodes an immunomodulating protein, or a composition having two nucleic acid molecules, one that comprises a nucleotide sequence that encodes a desired peptide or protein and one that comprises a nucleotide sequence that encodes an immunomodulating protein. The nucleic acid molecule(s) may be provided as plasmid DNA, the nucleic acid molecules of recombinant vectors or as part of the genetic material provided in an attenuated vaccine or cell vaccine. Alternatively, in some embodiments, the immunomodulating protein may be delivered as a protein.

According to some embodiments, combinations of two or more immunomodulating proteins are administered to an individual. In some embodiments, genes encoding a combination of two or more immunomodulating proteins are administered to an individual together with a gene that encodes an immunogen and/or an immunogenic protein as part of a vaccine protocol. In some embodiments, a combination of an immunomodulating protein and a gene encoding an immunomodulating proteins is administered to an individual together with a gene that encodes an immunogen and/or an immunogenic protein as part of a vaccine protocol. In some embodiments, a combination of two or more immunomodulating proteins is

administered to an individual together with a gene that encodes an immunogen and/or an immunogenic protein as part of a vaccine protocol.

According to some embodiments, immunomodulating proteins are administered to an individual in combination with the costimulatory molecule CD86 (B7.2). In some embodiments, genes encoding a combination of CD86 and one or more immunomodulating proteins are administered to an individual together with a gene that encodes an immunogen and/or an immunogenic protein as part of a vaccine protocol. In some embodiments, a combination of CD86 protein and a gene encoding an immunomodulating proteins is administered to an individual together with a gene that encodes an immunogen and/or an immunogenic protein as part of a vaccine protocol. In some embodiments, a combination of immunomodulating protein and a gene encoding CD86 protein is administered to an individual together with a gene that encodes an immunogen and/or an immunogenic protein as part of a vaccine protocol. In some embodiments, a combination of CD86 and one or more immunomodulating proteins is administered to an individual together with a gene that encodes an immunogen and/or an immunogenic protein as part of a vaccine protocol. In some embodiments, genes encoding a combination of CD86 and one or more chemokines and/or adhesion molecules are administered to an individual together with a gene that encodes an immunogen and/or an immunogenic protein as part of a vaccine protocol. In some embodiments, genes encoding a combination of CD86 and ICAM-1 are administered to an individual together with a gene that encodes an immunogen and/or an immunogenic protein as part of a vaccine protocol.

According to some aspects of the present invention, compositions and methods are provided which prophylactically and/or therapeutically immunize an individual against a pathogen or abnormal, disease-related cell. The genetic material that encodes a peptide or protein that shares at least an epitope with an immunogenic protein found on the pathogen or cells to be targeted and genetic material that encodes an immunomodulating protein. Alternatively, in some embodiments, the immunomodulating protein may be delivered as a protein.

The genetic material is expressed by the individual's cells and serves as an immunogenic target against which an immune response is elicited. The resulting

immune response is broad based: in addition to a humoral immune response, both arms of the cellular immune response are elicited. The methods of the present invention are useful for conferring prophylactic and therapeutic immunity. Thus, a method of immunizing includes both methods of immunizing against immunogens and thus for example of protecting an individual from pathogen challenge, or occurrence or proliferation of specific cells as well as methods of treating an individual suffering from pathogen infection, hyperproliferative disease or autoimmune disease.

As used herein the term "target protein" is meant to refer to peptides and protein encoded by gene constructs of the present invention which act as target proteins for an immune response. The term "target protein" and "immunogen' are used interchangeably and refer to a protein against which an immune response can be elicited. The target protein is an immunogenic protein which shares at least an epitope with a protein from the pathogen or undesirable cell-type such as a cancer cell or a cell involved in autoimmune disease against which immunization is required. The immune response directed against the target protein will protect the individual against and treat the individual for the specific infection or disease with which the target protein is associated.

The present invention is useful to elicit broad immune responses against a target protein, i.e. proteins specifically associated with pathogens, allergens or the individual's own "abnormal" cells. The present invention is useful to immunize individuals against pathogenic agents and organisms such that an immune response against a pathogen protein provides protective immunity against the pathogen. The present invention is useful to combat hyperproliferative diseases and disorders such as cancer by eliciting an immune response against a target protein that is specifically associated with the hyperproliferative cells. The present invention is useful to combat autoimmune diseases and disorders by eliciting an immune response against a target protein that is specifically associated with cells involved in the autoimmune condition.

According to some aspects of the present invention, DNA or RNA that encodes a target protein and an immunomodulating protein is introduced into the cells of tissue of an individual where it is expressed, thus producing the target protein. The DNA or RNA sequences encoding the target protein and immunomodulating protein are linked to regulatory elements necessary for expression in the cells of the individual. Regulatory

elements for DNA expression include a promoter and a polyadenylation signal. In addition, other elements, such as a Kozak region, may also be included in the genetic construct.

As used herein, the term "genetic construct" refers to the DNA or RNA molecules that comprise a nucleotide sequence which encodes the target protein and which includes initiation and termination signals operably linked to regulatory elements including a promoter and polyadenylation signal capable of directing expression in the cells of the vaccinated individual and/or a nucleotide sequence which encodes the immunomodulating protein and which includes initiation and termination signals operably linked to regulatory elements including a promoter and polyadenylation signal capable of directing expression in the cells of the vaccinated individual. In some embodiments, expressible forms sequences that encode the target protein and expressible forms sequences that encode an immunomodulating protein are found on the same nucleic acid molecule that is delivered to the individual. In some embodiments, expressible forms sequences that encode the target protein occur on separate same nucleic acid molecule from the nucleic acid molecules that contain expressible forms sequences that encode an immunomodulating protein. In such cases, both molecules are delivered to the individual.

As used herein, the term "expressible form" refers to gene constructs which contain the necessary regulatory elements operable linked to a coding sequence that encodes a target protein or an immunomodulating protein, such that when present in the cell of the individual, the coding sequence will be expressed.

As used herein, the term "sharing an epitope" refers to proteins which comprise at least one epitope that is identical to or substantially similar to an epitope of another protein.

As used herein, the term "substantially similar epitope" is meant to refer to an epitope that has a structure which is not identical to an epitope of a protein but nonetheless invokes an cellular or humoral immune response which cross reacts to that protein.

Genetic constructs may comprise a nucleotide sequence that encodes a target protein or an immunomodulating protein operably linked to regulatory elements

needed for gene expression. According to the invention, combinations of gene constructs which include one that comprises an expressible form of the nucleotide sequence that encodes a target protein and one that includes an expressible form of the nucleotide sequence that encodes an immunomodulating protein are provided. Incorporation into a living cell of the DNA or RNA molecule(s) which include the combination of gene constructs results in the expression of the DNA or RNA and production of the target protein and the immunomodulating protein. An enhanced immune response against the target protein results.

When taken up by a cell, the genetic construct(s) may remain present in the cell as a functioning extrachromosomal molecule and/or integrate into the cell's chromosomal DNA. DNA may be introduced into cells where it remains as separate genetic material in the form of a plasmid or plasmids. Alternatively, linear DNA which can integrate into the chromosome may be introduced into the cell. When introducing DNA into the cell, reagents which promote DNA integration into chromosomes may be added. DNA sequences which are useful to promote integration may also be included in the DNA molecule. Alternatively, RNA may be administered to the cell. It is also contemplated to provide the genetic construct as a linear minichromosome including a centromere, telomeres and an origin of replication. Gene constructs may remain part of the genetic material in attenuated live microorganisms or recombinant microbial vectors which live in cells. Gene constructs may be part of genomes of recombinant viral vaccines where the genetic material either integrates into the chromosome of the cell or remains extrachromosomal.

Genetic constructs include regulatory elements necessary for gene expression of a nucleic acid molecule. The elements include: a promoter, an initiation codon, a stop codon, and a polyadenylation signal. In addition, enhancers are often required for gene expression of the sequence that encodes the target protein or the immunomodulating protein. It is necessary that these elements be operable linked to the sequence that encodes the desired proteins and that the regulatory elements are operably in the individual to whom they are administered.

Initiation codons and stop codon are generally considered to be part of a nucleotide sequence that encodes the desired protein. However, it is necessary that these

elements are functional in the individual to whom the gene construct is administered. The initiation and termination codons must be in frame with the coding sequence.

Promoters and polyadenylation signals used must be functional within the cells of the individual.

Examples of promoters useful to practice the present invention, especially in the production of a genetic vaccine for humans, include but are not limited to promoters from Simian Virus 40 (SV40), Mouse Mammary Tumor Virus (MMTV) promoter, Human Immunodeficiency Virus (HIV) such as the HIV Long Terminal Repeat (LTR) promoter, Moloney virus, ALV, Cytomegalovirus (CMV) such as the CMV immediate early promoter, Epstein Barr Virus (EBV), Rous Sarcoma Virus (RSV) as well as promoters from human genes such as human Actin, human Myosin, human Hemoglobin, human muscle creatine and human metalothionein.

Examples of polyadenylation signals useful to practice the present invention, especially in the production of a genetic vaccine for humans, include but are not limited to bovine growth hormone polyadenylation signal, SV40 polyadenylation signals and LTR polyadenylation signals. In particular, the SV40 polyadenylation signal which is in pCEP4 plasmid (Invitrogen, San Diego CA), referred to as the SV40 polyadenylation signal, is used.

In addition to the regulatory elements required for DNA expression, other elements may also be included in the DNA molecule. Such additional elements include enhancers. The enhancer may be selected from the group including but not limited to: human Actin, human Myosin, human Hemoglobin, human muscle creatine and viral enhancers such as those from CMV, RSV and EBV.

Genetic constructs can be provided with mammalian origin of replication in order to maintain the construct extrachromosomally and produce multiple copies of the construct in the cell. Plasmids pCEP4 and pREP4 from Invitrogen (San Diego, CA) contain the Epstein Barr virus origin of replication and nuclear antigen EBNA-1 coding region which produces high copy episomal replication without integration. In some embodiments, the cDNA encoding the immunomodulating protein is inserted into pCDNA3.

In some preferred embodiments related to immunization applications, nucleic acid molecule(s) are delivered which include nucleotide sequences that encode a target protein, the immunomodulating protein and, additionally, genes for proteins which further enhance the immune response against such target proteins. Examples of such genes are those which encode other cytokines and lymphokines such as α-interferon, gamma-interferon, platelet derived growth factor (PDGF), TNF, epidermal growth factor (EGF), IL-1, IL-2, IL-4, IL-6, IL-8, IL-10 and IL-12. In some embodiments, it is preferred that the gene for GM-CSF is included in genetic constructs used in immunizing compositions.

An additional element may be added which serves as a target for cell destruction if it is desirable to eliminate cells receiving the genetic construct for any reason. A herpes thymidine kinase (tk) gene in an expressible form can be included in the genetic construct. The drug gangcyclovir can be administered to the individual and that drug will cause the selective killing of any cell producing tk, thus, providing the means for the selective destruction of cells with the genetic construct.

In order to maximize protein production, regulatory sequences may be selected which are well suited for gene expression in the cells the construct is administered into. Moreover, codons may be selected which are most efficiently transcribed in the cell. One having ordinary skill in the art can produce DNA constructs which are functional in the cells.

Routes of administration include, but are not limited to, intramuscular, intranasally, intraperitoneal, intradermal, subcutaneous, intravenous, intraarterially, intraoccularly and oral as well as topically, transdermally, by inhalation or suppository or to mucosal tissue such as by lavage to vaginal, rectal, urethral, buccal and sublingual tissue. Preferred routes of administration include to mucosal tissue, intramuscular, intraperitoneal, intradermal and subcutaneous injection. Genetic constructs may be administered by means including, but not limited to, traditional syringes, needleless injection devices, or "microprojectile bombardment gene guns".

The pharmaceutical compositions according to the present invention comprise about 1 nanogram to about 2000 micrograms of DNA. In some preferred embodiments, pharmaceutical compositions according to the present invention comprise

about 5 nanogram to about 1000 micrograms of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 10 nanograms to about 800 micrograms of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 0.1 to about 500 micrograms of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 1 to about 350 micrograms of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 25 to about 250 micrograms of DNA. In some preferred embodiments, the pharmaceutical compositions contain about 100 to about 200 micrograms DNA.

The pharmaceutical compositions according to the present invention are formulated according to the mode of administration to be used. In cases where pharmaceutical compositions are injectable pharmaceutical compositions, they are sterile, pyrogen free and particulate free. An isotonic formulation is preferably used. Generally, additives for isotonicity can include sodium chloride, dextrose, mannitol, sorbitol and lactose. In some cases, isotonic solutions such as phosphate buffered saline are preferred. Stabilizers include gelatin and albumin. In some embodiments, a vaso-constriction agent is added to the formulation.

In some embodiments, the nucleic acid molecule is delivered to the cells in conjunction with administration of a polynucleotide function enhancer or a genetic vaccine facilitator agent. Polynucleotide function enhancers are described in U.S. Serial Number 08/008,342 filed January 26, 1993, U.S. Serial Number 08/029,336 filed March 11, 1993, U.S. Serial Number 08/125,012 filed September 21, 1993, and International Application Serial Number PCT/US94/00899 filed January 26, 1994, which are each incorporated herein by reference. Genetic vaccine facilitator agents are described in U.S. Serial Number 08/221,579 filed April 1, 1994, which is incorporated herein by reference. The co-agents which are administered in conjunction with nucleic acid molecules may be administered as a mixture with the nucleic acid molecule or administered separately simultaneously, before or after administration of nucleic acid molecules. In addition, other agents which may function transfecting agents and/or replicating agents and/or inflammatory agents and which may be co-administered with a GVF include growth factors, cytokines and lymphokines such as α-interferon, gamma-interferon, platelet derived growth factor (PDGF), TNF, epidermal growth factor (EGF), IL-1, IL-2, IL-4,

IL-6, IL-8, IL-10 and IL-12 as well as fibroblast growth factor, surface active agents such as immune-stimulating complexes (ISCOMS), Freund's incomplete adjuvant, LPS analog including monophosphoryl Lipid A (MPL), muramyl peptides, quinone analogs and vesicles such as squalene and squalene, and hyaluronic acid may also be used administered in conjunction with the genetic construct. In some embodiments, an immunomodulating protein may be used as a GVF.

Nucleic acid molecules which are delivered to cells according to the invention may serve as genetic templates for proteins that function as prophylactic and/or therapeutic immunizing agents. In preferred embodiments, the nucleic acid the nucleic acid molecules comprise the necessary regulatory sequences for transcription and translation of the coding region in the cells of the animal.

The present invention may be used to immunize an individual against all pathogens such as viruses, prokaryote and pathogenic eukaryotic organisms such as unicellular pathogenic organisms and multicellular parasites. The present invention is particularly useful to immunize an individual against those pathogens which infect cells and which are not encapsulated such as viruses, and prokaryote such as gonorrhoea, listeria and shigella. In addition, the present invention is also useful to immunize an individual against protozoan pathogens which include a stage in the life cycle where they are intracellular pathogens. As used herein, the term "intracellular pathogen" is meant to refer to a virus or pathogenic organism that, at least part of its reproductive or life cycle, exists within a host cell and therein produces or causes to be produced, pathogen proteins. Table 2 provides a listing of some of the viral families and genera for which vaccines according to the present invention can be made. DNA constructs that comprise DNA sequences which encode the peptides that comprise at least an epitope identical or substantially similar to an epitope displayed on a pathogen antigen such as those antigens listed on the tables are useful in vaccines. Moreover, the present invention is also useful to immunize an individual against other pathogens including prokaryotic and eukaryotic protozoan pathogens as well as multicellular parasites such as those listed on Table 3.

In order to produce a genetic vaccine to protect against pathogen infection, genetic material which encodes immunogenic proteins against which a protective immune response can be mounted must be included in a genetic construct as

the coding sequence for the target. Whether the pathogen infects intracellularly, for which the present invention is particularly useful, or extracellularly, it is unlikely that all pathogen antigens will elicit a protective response. Because DNA and RNA are both relatively small and can be produced relatively easily, the present invention provides the additional advantage of allowing for vaccination with multiple pathogen antigens. The genetic construct used in the genetic vaccine can include genetic material which encodes many pathogen antigens. For example, several viral genes may be included in a single construct thereby providing multiple targets.

Tables 2 and 3 include lists of some of the pathogenic agents and organisms for which genetic vaccines can be prepared to protect an individual from infection by them. In some preferred embodiments, the methods of immunizing an individual against a pathogen are directed against HIV, HTLV or HBV.

Another aspect of the present invention provides a method of conferring a broad based protective immune response against hyperproliferating cells that are characteristic in hyperproliferative diseases and to a method of treating individuals suffering from hyperproliferative diseases. As used herein, the term "hyperproliferative diseases" is meant to refer to those diseases and disorders characterized by hyperproliferation of cells. Examples of hyperproliferative diseases include all forms of cancer and psoriasis.

It has been discovered that introduction of a genetic construct that includes a nucleotide sequence which encodes an immunogenic "hyperproliferating cell"-associated protein into the cells of an individual results in the production of those proteins in the vaccinated cells of an individual. As used herein, the term "hyperproliferative-associated protein" is meant to refer to proteins that are associated with a hyperproliferative disease. To immunize against hyperproliferative diseases, a genetic construct that includes a nucleotide sequence which encodes a protein that is associated with a hyperproliferative disease is administered to an individual.

In order for the hyperproliferative-associated protein to be an effective immunogenic target, it must be a protein that is produced exclusively or at higher levels in hyperproliferative cells as compared to normal cells. Target antigens include such proteins, fragments thereof and peptides which comprise at least an epitope found on

such proteins. In some cases, a hyperproliferative-associated protein is the product of a mutation of a gene that encodes a protein. The mutated gene encodes a protein which is nearly identical to the normal protein except it has a slightly different amino acid sequence which results in a different epitope not found on the normal protein. Such target proteins include those which are proteins encoded by oncogenes such as *myb, myc, fyn,* and the translocation gene *bcr/abl, ras, src,* P53, *neu, trk* and EGRF. In addition to oncogene products as target antigens, target proteins for anti-cancer treatments and protective regimens include variable regions of antibodies made by B cell lymphomas and variable regions of T cell receptors of T cell lymphomas which, in some embodiments, are also used target antigens for autoimmune disease. Other tumor-associated proteins can be used as target proteins such as proteins which are found at higher levels in tumor cells including the protein recognized by monoclonal antibody 17-1A and folate binding proteins.

While the present invention may be used to immunize an individual against one or more of several forms of cancer, the present invention is particularly useful to prophylactically immunize an individual who is predisposed to develop a particular cancer or who has had cancer and is therefore susceptible to a relapse. Developments in genetics and technology as well as epidemiology allow for the determination of probability and risk assessment for the development of cancer in individual. Using genetic screening and/or family health histories, it is possible to predict the probability a particular individual has for developing any one of several types of cancer.

Similarly, those individuals who have already developed cancer and who have been treated to remove the cancer or are otherwise in remission are particularly susceptible to relapse and reoccurrence. As part of a treatment regimen, such individuals can be immunized against the cancer that they have been diagnosed as having had in order to combat a recurrence. Thus, once it is known that an individual has had a type of cancer and is at risk of a relapse, they can be immunized in order to prepare their immune system to combat any future appearance of the cancer.

The present invention provides a method of treating individuals suffering from hyperproliferative diseases. In such methods, the introduction of genetic constructs

serves as an immunotherapeutic, directing and promoting the immune system of the individual to combat hyperproliferative cells that produce the target protein.

The present invention provides a method of treating individuals suffering from autoimmune diseases and disorders by conferring a broad based protective immune response against targets that are associated with autoimmunity including cell receptors and cells which produce "self"-directed antibodies.

T cell mediated autoimmune diseases include Rheumatoid arthritis (RA), multiple sclerosis (MS), Sjogren's syndrome, sarcoidosis, insulin dependent diabetes mellitus (IDDM), autoimmune thyroiditis, reactive arthritis, ankylosing spondylitis, scleroderma, polymyositis, dermatomyositis, psoriasis, vasculitis, Wegener's granulomatosis, Crohn's disease and ulcerative colitis. Each of these diseases is characterized by T cell receptors that bind to endogenous antigens and initiate the inflammatory cascade associated with autoimmune diseases. Vaccination against the variable region of the T cells would elicit an immune response including CTLs to eliminate those T cells.

In RA, several specific variable regions of T cell receptors (TCRs) which are involved in the disease have been characterized. These TCRs include Vβ-3, Vβ-14, Vβ-17 and Vα-17. Thus, vaccination with a DNA construct that encodes at least one of these proteins will elicit an immune response that will target T cells involved in RA. See: Howell, M.D., *et al.*, 1991 *Proc. Natl. Acad. Sci. USA* **88**:10921-10925; Paliard, X., *et al.*, 1991 *Science* **253**:325-329; Williams, W.V., *et al.*, 1992 *J. Clin. Invest.* **90**:326-333; each of which is incorporated herein by reference.

In MS, several specific variable regions of TCRs which are involved in the disease have been characterized. These TCRs include Vβ-7 and Vα-10. Thus, vaccination with a DNA construct that encodes at least one of these proteins will elicit an immune response that will target T cells involved in MS. See: Wucherpfennig, K.W., *et al.*, 1990 *Science* **248**:1016-1019; Oksenberg, J.R., *et al.*, 1990 *Nature* **345**:344-346; each of which is incorporated herein by reference.

In scleroderma, several specific variable regions of TCRs which are involved in the disease have been characterized. These TCRs include Vβ-6, Vβ-8, Vβ-14 and Vα-16, Vα-3C, Vα-7, Vα-14, Vα-15, Vα-16, Vα-28 and Vα-12. Thus, vaccination

with a DNA construct that encodes at least one of these proteins will elicit an immune response that will target T cells involved in scleroderma.

In order to treat patients suffering from a T cell mediated autoimmune disease, particularly those for which the variable region of the TCR has yet to be characterized, a synovial biopsy can be performed. Samples of the T cells present can be taken and the variable region of those TCRs identified using standard techniques. Genetic vaccines can be prepared using this information.

B cell mediated autoimmune diseases include Lupus (SLE), Grave's disease, myasthenia gravis, autoimmune hemolytic anemia, autoimmune thrombocytopenia, asthma, cryoglobulinemia, primary biliary sclerosis and pernicious anemia. Each of these diseases is characterized by antibodies which bind to endogenous antigens and initiate the inflammatory cascade associated with autoimmune diseases. Vaccination against the variable region of antibodies would elicit an immune response including CTLs to eliminate those B cells that produce the antibody.

In order to treat patients suffering from a B cell mediated autoimmune disease, the variable region of the antibodies involved in the autoimmune activity must be identified. A biopsy can be performed and samples of the antibodies present at a site of inflammation can be taken. The variable region of those antibodies can be identified using standard techniques. Genetic vaccines can be prepared using this information.

In the case of SLE, one antigen is believed to be DNA. Thus, in patients to be immunized against SLE, their sera can be screened for anti-DNA antibodies and a vaccine can be prepared which includes DNA constructs that encode the variable region of such anti-DNA antibodies found in the sera.

Common structural features among the variable regions of both TCRs and antibodies are well known. The DNA sequence encoding a particular TCR or antibody can generally be found following well known methods such as those described in Kabat, *et al.* 1987 *Sequence of Proteins of Immunological Interest* U.S. Department of Health and Human Services, Bethesda MD, which is incorporated herein by reference. In addition, a general method for cloning functional variable regions from antibodies can be found in Chaudhary, V.K., *et al.*, 1990 *Proc. Natl. Acad. Sci. USA* **87**:1066, which is incorporated herein by reference.

In addition to using expressible forms of immunomodulating protein coding sequence to improve genetic vaccines, the present invention relates to improved attenuated live vaccines and improved vaccines which use recombinant vectors to deliver foreign genes that encode antigens. Examples of attenuated live vaccines and those using recombinant vectors to deliver foreign antigens are described in U.S. Patent Nos.: 4,722,848; 5,017,487; 5,077,044; 5,110,587; 5,112,749; 5,174,993; 5,223,424; 5,225,336; 5,240,703; 5,242,829; 5,294,441; 5,294,548; 5,310,668; 5,387,744; 5,389,368; 5,424,065; 5,451,499; 5,453,364; 5,462,734; 5,470,734; and 5,482,713, which are each incorporated herein by reference. Gene constructs are provided which include the nucleotide sequence that encodes an immunomodulating protein is operably linked to regulatory sequences that can function in the vaccinee to effect expression. The gene constructs are incorporated in the attenuated live vaccines and recombinant vaccines to produce improved vaccines according to the invention.

The present invention provides an improved method of immunizing individuals that comprises the step of delivering gene constructs to the cells of individuals as part of vaccine compositions which include are provided which include DNA vaccines, attenuated live vaccines and recombinant vaccines. The gene constructs comprise a nucleotide sequence that encodes an immunomodulating protein and that is operably linked to regulatory sequences that can function in the vaccinee to effect expression. The improved vaccines result in an enhanced cellular immune response.

Another aspect of the present invention relates to the use of either GM-CSF or a nucleic acid molecule encoding GM-CSF or both in combination with a DNA vaccine for which a strong antibody response or helper T cell response is particularly desirable. One example of such a vaccine is a vaccine against hepatitis B. Other examples include extracellular pathogens and allergens. The administration of either GM-CSF or a nucleic acid molecule encoding GM-CSF or both in combination with a DNA vaccine is also useful for vaccinated individuals identified as being immunocompromised.

Another embodiment of the present invention relates to the use of anti-chemokine antibodies to treat patients who have autoimmune diseases. Autoimmune diseases are outlined above. Anti-chemokine antibodies include antibodies specific for

MCP-1, MIP-1α, MIP-1β, IL-8 or RANTES. Anti-chemokine antibodies may be administered to patients suspected of suffering form such diseases in therapeutically effective amounts to reduce or alleviate symptoms.

Pharmaceutical compositions for treating autoimmune disease comprise an antibody specific for a chemokine and a pharmaceutically acceptable carrier. According to preferred embodiments, the compositions are injectable. The sterile, pyrogen-free, particulate-free injectable compositions comprise one or more an antibody specific for a chemokine and a pharmaceutically acceptable carrier or injection vehicle.

The antibodies are made according to conventional methods for producing monoclonal antibodies. The carrier be selected from those well known to persons having ordinary skill in the art. An example of a carrier is sterile saline.

Those having ordinary skill in the art can produce monoclonal antibodies which specifically bind to a MCP-1, MIP-1α, MIP-1β, IL-8 or RANTES using standard techniques and readily available starting materials. The techniques for producing monoclonal antibodies are outlined in Harlow, E. and D. Lane, (1988) *ANTIBODIES: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor NY, which is incorporated herein by reference, provide detailed guidance for the production of hybridomas and monoclonal antibodies which specifically bind to target proteins.

Briefly, the chemokine is injected into mice. The spleen of the mouse is removed, the spleen cells are isolated and fused with immortalized mouse cells. The hybrid cells, or hybridomas, are cultured and those cells which secrete antibodies are selected. The antibodies are analyzed and, if found to specifically bind to the protein of interest, the hybridoma which produces them is cultured to produce a continuous supply of antigen specific antibodies.

According to the present invention, antibodies specific for MCP-1, MIP-1α, MIP-1β, IL-8 or RANTES may be used to treat an autoimmune disease. Accordingly, MCP-1, MIP-1α, MIP-1β, IL-8 or RANTES is used to generate hybridomas. The genes which encode these proteins are widely known and readily available to those having ordinary skill in the art. Thus, one having ordinary skill in the art can make antibodies useful to practice the present invention. In addition to rodent antibodies, the present invention relates to human antibodies, humanized antibodies,

Fabs and chimeric antibodies and Fabs which bind to MCP-1, MIP-1α, MIP-1β, IL-8 or RANTES which may be produced routinely by those having ordinary skill in the art.

Those having ordinary skill in the art can readily identify individuals who suffer form or are susceptible to an autoimmune disease.

The compositions may include additional components to render them more effective. For example, a composition of the invention may comprise multiple anti-chemokine antibodies including antibodies specific for different chemokines and antibodies specific for different epitopes of the same chemokine.

About 5 μg to 5000 mg of antibody may be administered. In some preferred embodiments, 50 μg to 500 mg of antibody may be administered. In other preferred embodiments, 500 μg to 50 mg of antibody may be administered. In a preferred embodiment, 5 mg of antibody is administered.

Compositions may be administered by an appropriate route such as, for example, by oral, intranasal, intramuscular, intraperitoneal or subcutaneous administration. In some embodiments, intravenous administration is preferred.

Subsequent to initial administration, individuals may be boosted by readministration. In some preferred embodiments, multiple administrations are performed.

EXAMPLES

Example 1

Introduction

To molecularly dissect the specific roles of chemokines in immune response we cloned cDNAs encoding the α-chemokine IL-8 as well as cDNAs encoding the β-chemokines MIP-1α, RANTES, and MCP-1 individually into expression vectors and co-immunized them along with DNA immunogens which encodes for HIV-1envelope or gag/pol proteins. Using these DNA vaccine constructs as model antigens, we examined the specific roles of the expression of chemokine genes play in the development of the immune responses through analyzing the antigen-specific humoral and cell-mediated immune responses induced following such immunization. We

observed that chemokines had specific, identifiable roles in the activation and modulation of antigen-specific immune responses.

Results

Induction of chemokines by DNA vaccination

Mice were immunized with 50 μg of pCDNA3 (control), pCEnv, or pCGag/pol. After two weeks, the mice were sacrificed, their spleens were harvested, and their lymphocytes were isolated. These cells were stimulated *in vitro* by antigen-specific stimulation (using fixed recombinant vaccinia infected stimulator cells) for 5 days. We collected the culture supernatant from the effector cells and tested them for the release of chemokines MIP1-α, MIP1-β, and RANTES. We observed that DNA immunization with pCEnv or pCGag/pol induced significantly greater levels of expression of β-chemokines MIP1-α, MIP1-β, and RANTES over those of control vector. The increase was present as early as 2 weeks following the first immunization, suggesting that β chemokines could be modulating immune responses *in vivo*. To determine the effects of the chemokines on antigen specific responses we next investigated their effects on immune responses induced by the DNA vaccine.

Construction of chemokine expression cassettes

The genes for chemokines IL-8, MIP1-α, MCP-1α, MCP-1, and RANTES were individually cloned into pCDNA3 plasmid expression vectors using methods described in Kim, J.J., D.B. Weiner (1997) Springer Sem Immunopathol *19* 174-195; Kim, J.J., et al. (1997) Nature Biot. *15*, 641-645; and Kim, J.J., et al. (1997) J. Immunol. *158*, 816-826, which are each incorporated herein by reference. These chemokine expression cassettes were verified by sequencing analysis of the entire insert (including both 5' and 3' flanking sequences). In addition, these chemokine constructs were transfected *in vitro* into RD cells and the expression of these constructs were verified by immunoprecipitation using relevant antibodies or by chemokine ELISA. The expression constructs for IL-8, MIP1-α, MCP-1, and RANTES were also used as vaccines and immunized into mice. It was determined by the *in vivo* expression technique described in Materials and Methods that these constructs expressed their encoded chemokines *in vivo* in mouse muscle tissue.

IL-8 is a strong inducer of T helper response